DOI:
10.1523/JNEUROSCI.0248-16.2016

Document Version
Peer reviewed version

The Journal of Neuroscience

http://jneurosci.msubmit.net

JN-RM-0248-16R1

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Commercial Interest:

Compartmentalized PDE4A5 signaling impairs hippocampal synaptic plasticity and long-term memory

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Number of figures: 5
Number of words in abstract: 263
Number of words in significant statement: 120
Number of words in the introduction: 650
Number of pages: 32
Running title: PDE4A5 signaling, memory and synaptic plasticity
Keywords: hippocampus, cAMP, PDE, phosphodiesterase, memory, LTP, FRET
Conflict of interest: The authors declare they have no conflict of interest.

Acknowledgements: We thank other Abel lab members for their help with these experiments and for their comments on the manuscript. We thank Jin Zhang for providing pICUE3. This research was supported by the Netherlands Organization for Scientific Research NWO-Rubicon Grant 825.07.029 (to R.H.), the University of Pennsylvania Research Foundation (to R.H. and T.A.), National Institutes of Health 1R01MH086415 9 (to T.A.), AG017628 (to T.A., A.I. Pack PI), 5K12GM081529 (to J.C.T., Y. Paterson PI), 5R01DK087650 (to S.R.N.Z.), and by Systems Biology Center of New York PSDGM071558 (to S.R.N.Z.). G.S.B. is supported by the MRC (MR/J007412/1). R.H. is supported by the Einsteinium Foundation. T.A. is the Brush Family Professor of Biology at the University of Pennsylvania.

Abstract

Alterations in cAMP signaling are thought to contribute to neurocognitive and neuropsychiatric disorders. Members of the cAMP-specific phosphodiesterase 4 (PDE4) family, which contains more than 25 different isoforms, play a key role in determining spatial cAMP degradation so as to orchestrate compartmentalized cAMP signaling in cells. Each isoform binds to a different set of protein complexes through its unique N-terminal domain, thereby leading to targeted degradation of cAMP in specific intracellular compartments. However the functional role of specific compartmentalized PDE4 isoforms has not been examined *in vivo*. Here, we show that increasing protein levels of the PDE4A5 isoform in hippocampal excitatory neurons impairs a long-lasting form of hippocampal synaptic plasticity and attenuates hippocampus-dependent long-term memories without affecting anxiety. In contrast, viral expression of a truncated version of PDE4A5, which lacks the unique N-terminal targeting domain, does not affect long-term memory. Further, overexpression of the PDE4A1 isoform, which targets a different subset of signalosomes, leaves memory undisturbed. Fluorescence resonance energy transfer (FRET) sensor-based cAMP measurements reveal that the full-length PDE4A5, in contrast to the truncated form, hampers forskolin-mediated increases in neuronal cAMP levels. Our study indicates that the unique N-terminal localization domain of PDE4A5 plays an essential role in the targeting of specific cAMP-dependent signaling critical for synaptic plasticity and memory. The development of compounds to disrupt the compartmentalization of individual PDE4 isoforms by targeting their unique N-terminal domains may provide a fruitful approach to prevent cognitive deficits in neuropsychiatric and neurocognitive disorders that are associated with alterations in cAMP signaling.

68 **Significance statement**

69 Neuronal signaling events are orchestrated with high spatial and temporal resolution.
70 Phosphodiesterase-4 (PDE4) isoforms target unique signaling complexes through their isoform-
71 specific N-terminal domain to enable cAMP degradation within specific cellular compartments.
72 The importance of the isoform-specific N-terminal targeting domain is highlighted here, where
73 we virally overexpress either full-length or truncated PDE4A5 lacking the N-terminal domain.
74 Overexpression of the full-length PDE4A5 isoform in hippocampal neurons negatively impacts
75 synaptic plasticity and memory, whereas expression of the truncated version does not alter
76 memory processes. Our work identifies PDE4A5 as a constraint on memory storage and paves
77 the way for novel therapeutic approaches to treat memory-related disorders by targeting the
78 unique N-terminal domain of individual PDE4 isoforms to regulate spatially constrained cAMP
79 signaling.

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Introduction

The cAMP signaling pathway plays an essential role in synaptic plasticity and memory (Havekes and Abel, 2009). Local degradation of cAMP within specific intracellular compartments is precisely orchestrated by cAMP-degrading phosphodiesterases (PDE) (Houslay, 2010; Mika et al., 2012). Isoforms of the PDE4 subfamily have a prominent role in regulating cAMP signaling in the mammalian brain (Houslay and Adams, 2003; O'donnell and Zhang, 2004; Sanderson and Sher, 2013), in *Drosophila* (Conti and Jin, 1999), and in the *Aplysia* nervous system (Park et al., 2005). Although the catalytic cAMP-degrading domain of the PDE4 isoforms is highly conserved, all of the more than 25 described isoforms have a unique N-terminal domain that targets each isoform to specific intracellular compartments where it binds to a unique set of protein complexes (Houslay, 2010). Pharmacological suppression of PDE4 activity promotes synaptic plasticity and memory (Randt et al., 1982; Barad et al., 1998; Rutten et al., 2009; Werenicz et al., 2012). The PDE4 selective inhibitor, rolipram, prevents memory deficits associated with sleep loss (Vecsey et al., 2009), traumatic brain injury (Titus et al., 2013), aging (Wimmer et al., 2011), muscarinic or NMDA receptor blockade (Egawa et al., 1997; Zhang and O'Donnell, 2000; Wiescholleck and Manahan-Vaughan, 2012), and mouse models of Alzheimer's disease (Gong et al., 2004; Reneerkens et al., 2009; Richter et al., 2013). However, the clinical use of broad PDE4 inhibitors is limited due to undesirable side effects including emesis and diarrhea (Zeller et al., 1984; Bertolino et al., 1988; Spina, 2008). To circumvent these issues, which compromise the clinical use of pan-PDE4 inhibitors, studies are needed to identify which PDE4 isoforms target signaling complexes critical for cognitive processes and then to exploit such data in order to develop isoform-specific inhibitor approaches.

The PDE4 family is encoded by 4 genes (PDE4A-D) (Houslay, 2010). PDE4B knockout mice perform normally in hippocampus-dependent learning paradigms (Siuciak et al., 2008), but display an anxiogenic-like phenotype (Zhang et al., 2008), a phenotype also observed in mice lacking PDE4A isoforms (Hansen et al., 2014). Importantly, recent work revealed that the PDE4A family is not involved in emesis (Hansen et al., 2014), in contrast to the PDE4D sub-family (Robichaud et al., 2002). Mice lacking all PDE4D isoforms display either memory enhancements or impairments depending on the task used (Rutten et al., 2008; Li et al., 2011). Although these mutant mouse models provide insight into the role of PDE4 families in plasticity and memory, they lack the resolution needed to identify which individual isoforms orchestrate the observed behavioral and cognitive phenotypes.

The importance of studying individual PDE family members is underscored by the complex regulation of isoform expression, which includes multiple promoters, alternative mRNA splicing and alternative translational initiation sequences (Houslay et al., 2007). Expression levels of individual PDE4 isoforms are altered in traumatic brain injury (Oliva et al., 2012), autism spectrum disorders, schizophrenia, and bipolar disorder (Braun et al., 2007; Fatemi et al., 2008b; Fatemi et al., 2008a), by electroconvulsive shock delivery, and treatment with antidepressants (Takahashi et al., 1999; D'Sa et al., 2005). Changes in cerebellar expression levels of PDE4A4, the human orthologue of rodent PDE4A5, are observed in patients with autism or bipolar disorder (Braun et al., 2007; Fatemi et al., 2008a). Further, PDE4A4/5 levels are upregulated in lung tissue of patients with chronic obstructive pulmonary disease (COPD) (Barber et al., 2004). Interestingly, COPD patients frequently suffer from sleep loss (Crinion and McNicholas, 2014), a condition that elevates PDE4A5 protein expression in the mouse hippocampus without affecting levels of other PDE4 isoforms (Vecsey et al., 2009). Although

these findings suggest that memory impairments may be associated with altered PDE4A4/5 expression, it is unclear whether PDE4A4/5 in healthy conditions targets signaling mechanisms critical for memory. In the present study, we used a viral approach to increase the mouse PDE4A5 protein levels selectively in hippocampal excitatory neurons *in vivo* to assess the impact of elevated PDE4A5 expression on hippocampal synaptic plasticity and memory.

Methods

Subjects

C57BL/6J male mice (2-3 months of age) were obtained from Jackson Laboratories at an age of 6 weeks and housed in groups of 4 with littermates on a 12 hr/12 hr light/dark schedule with lights on at 7 am (ZT 0). Mice had food and water available *ad libitum*. Mice underwent surgery, at an age of 8-12 weeks, were individually housed for 5 days to recover from surgery and then pair-housed throughout the experiment. For all behavioral experiments, mice were handled in the experimental room for 5 days for 1 minute per day. All experiments were conducted according to National Institutes of Health guidelines for animal care and use and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Viral injections

The AAVs were injected using a nanofil 33G beveled needles (WPI) attached to a 10 µl Hamilton syringe controlled by a microsyringe pump (UMP3; WPI) at the following sites (A/P -1.9 mm, D/V +/- 1.5 mm, and 1.5 mm below bregma) as described previously (Havekes et al., 2014). The needle was slowly lowered to the target site over the course of 3 minutes and remained at the target site for 1 minute before beginning of the injection (0.2 µl per minute). Approximately 1 µl (corrected for genome copy number between constructs) was injected per hippocampus. After the injection, the needle remained at the target site for 1 minute and then was slowly removed over a 5-minute period.

DNA manipulation and Virus constructs

Full-length rat PDE4A5 and mouse PDE4A1 constructs were based on Genbank accession numbers L27057.1 and M26715. The truncated PDE4A5 that lacks the first 303 base pairs

encoding the isoform unique N-terminal domain (Bolger et al., 2003) (referred to as PDE4A5$^{\Delta 4}$) was generated using standard PCR cloning procedures and the Stratagene PfuUltra High-Fidelity DNA polymerase. The pAAV$_9$-CaMKIIα0.4-PDE4A5-VSV, pAAV$_9$-CaMKIIα0.4-PDE4A5$^{\Delta 4}$-HA, pAAV$_9$-CaMKIIα0.4-PDE4A1-HA, and pAAV$_9$-CaMKIIα0.4-eGFP were produced through standard methods and packaged by the University of Pennsylvania viral core. Titers ranged from 1.06 x 10^{13} to 2.02 x 10^{13} genome copy numbers. A 0.4kb CaMKIIα promoter fragment (Dittgen et al., 2004) was used to drive expression selectively in excitatory neurons.

Biochemistry

ELISA-based assay kits (ENZO Life Sciences) were used to measure cAMP content following the manufacturer's instructions. PDE activity assays and western blots were conducted as described (Vecsey et al., 2009). For all other protein analyses, tissue lysates were prepared in Tris 50 mM, pH: 9, Sodium deoxycholate 1 %, Sodium fluoride 50 mM, activated sodium vanadate 20 µM, EDTA 20 µM, and Beta-Glycerophosphate 40 µM. Protease inhibitors (Roche) and phosphatase inhibitors (Thermo Scientific) were added to the freshly prepared buffer just prior to tissue lysations. Samples were centrifuged for 10 minutes at 13000 x g at 4°C and supernatant was collected. Protein concentration of the samples was measured using the Bradford method. LDS sample buffer (Nupage, Invitrogen) including β-mercaptoethanol was added and samples were for boiled for 5 minutes prior to loading on Criterion TGX 18-well 4-20 % gels (BioRad). After electrophoreses, proteins were transferred to PVDF membrane followed by blocking for 1 hr in 5% milk in TBST. After blocking, membranes were incubated in 5% milk or BSA with one of the following antibodies were used GAPDH (Santa Cruz, K1511, 1:1000), PDE4A (1:1000 (Vecsey et al., 2009)), PDE4A5 (1:1000 (Vecsey et al., 2009)), HA-tag (Roche, 1867423, 1:1000), VSV-tag (Abcam, 1874, 1:3000). After incubation with the primary

antibodies, membranes were incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature (Santa Cruz mouse secondary antibody, sc-2318, 1:1000; Santa Cruz; rabbit secondary antibody, sc-2030, 1:5000; Santa Cruz rat secondary antibody, sc-2032, 1:1000). The immunoreactive bands were captured on autoradiography film (Kodak) and analyzed using ImageJ (NIH).

Immunohistochemistry

Immunohistochemistry was conducted as described (Isiegas et al., 2008). The following antibodies or combinations of antibodies were used; PDE4A5 (1:200 (Vecsey et al., 2009)), HA-tag (Roche, 1867423, 1:200), VSV-tag (Abcam, 1874, 1:2000), GFAP-alexa 488 (Millipore, 43202, 1:300), followed by the appropriate Alexa fluor-conjugated secondary antibodies (1:1000 Invitrogen) for 1 hr at room temperature. Images were captured using a Leica SP8 confocal microscope. Immunolabeling for cAMP was conducted using cAMP antibodies (Millipore, 07-1497, 1:200) in combination with the DAB procedure (Havekes et al., 2007). Optical density measurements in the corpus callosum were used to correct for background labeling. DAB images were captured using a Fisher Scientific light microscope and optical density measurements were conducted using ImageJ.

Behavioral assays

The object-place recognition task, fear conditioning task, open field task and zero maze task were conducted as described (Tretter et al., 2009; Oliveira et al., 2010; Havekes et al., 2012b; Havekes et al., 2014). Training and testing was conducted between ZT0 and ZT4.

Cell-culture studies

Neurons (100,000 per ml of C57BL/6J mouse hippocampus) were plated in a 24-well dish with coated coverslips. A week later, we transduced the neurons with adeno-associated virus (AAV) vectors expressing VSV-tagged PDE4A5 (PDE4A5-VSVg) and HA-tagged PDE4A5 N-terminal truncated mutant (PDE4A5delta4-HA). At seven days post-transduction, the neuronal cells were fixed with 4% paraformaldehyde (PFA) in PBS and incubated with anti-VSV (ab1874; Abcam) and anti-HA (ab130275: Abcam) antibodies for immunofluorescence analysis. The neuron samples were visualized with secondary antibodies conjugated with Alexa fluor 488 and 568 dyes (Thermo Fisher Scientific) using fluorescence imaging microscope using GFP- and RFP-selective filter sets.

Electrophysiology

Field recordings in hippocampal slices were conducted as previously described (Havekes et al., 2012b).

Fret sensor imaging

cAMP was measured using the ICUE3 biosensor as described previously (DiPilato and Zhang, 2009). Primary rat hippocampal cultures were obtained as previously described (Neves et al., 2008). Primary hippocampal cultures (DIV 8-12) were transfected using Lipofectamine 2000 (Life technologies) with pICUE3 (kindly provided by Jin Zhang) and mCherry alone, or pPDE4A5-full-mcherry, or pPDE4A5-truncated-mcherry. 24 hours later, neurons were pre-incubated for 60 min at 37 °C with nifedipine (Sigma-Aldrich) and tetrodotoxin (Tocris) to eliminate any calcium or depolarization-induced signaling. Neurons were then transferred to an imaging chamber maintained at 32 °C and continually perfused with 1X HBSS (Life Technologies) containing 25 mM HEPES buffer (Life Technologies), 10 mM glucose, and 0.5

mM Trolox (Sigma-Aldrich). Multiple ICUE3 and mCherry expressing neurons were selected. Transfected neurons were imaged every 60 sec on an inverted Axio-Observer Z1 microscope (Zeiss) with an automated stage using AxioVision 4.8 software using a Plan-Apochromat 40X/1.3 oil objective and a quantEM electron-multiplying charge-coupled device camera (Photometrics), illuminated by a Colibri controlled LED system (Zeiss). At each timepoint, images were acquired from the Cerulean and FRET channels with the following settings: (ex) LED 455 nm and (em) 475/40 nm for Cerulean; and (ex) LED 455 nm and (em) 535/25 nm for FRET. The time course of the experiment contained three phases: 5-7 min baseline, 25 min of 50 μM of Forskolin (FK; Tocris) followed by 10 min 100 μM of 3-isobutyl-1-methylxantine (IBMX; Tocris) to saturate cAMP response. Images were converted into gray scale values and the dendritic fluorescent intensities were measured with ImageJ after background correction. The ratio of Cerulean and FRET was calculated for each time point.

Statistical analyses

Data analysis was performed using SPSS (version 20). Data was analyzed using one-way or two-way ANOVAs (in some cases with repeated measures as the within subject variable), independent samples t-tests, paired t-tests, or one-way ANOVAs combined with Tukey posthoc tests. Differences were considered statistically significant when $P < 0.05$. All data are plotted as mean ± s.e.m.

268 **Results**

269 *Overexpression of PDE4A5 in hippocampal excitatory neurons increases PDE4 activity and*
270 *reduces cAMP levels*

Virally-induced PDE4A5 expression was observed in excitatory neurons throughout the hippocampus (Fig. 1A-E). Double labeling studies using GFAP as a marker for astrocytes suggested that expression was restricted to neurons (Fig. 1F-H). Biochemical analyses confirmed the virally-induced increase in PDE4A5 protein levels in hippocampal lysates (eGFP, n = 4; PDE4A5, n = 4; t-test$_{(7)}$ = -6.84, P = 0.001; Fig. 1I) and elevated PDE4 activity (eGFP, n = 10; PDE4A5, n = 9; t-test$_{(17)}$ = 13.67, P = 0.0001; Fig. 1J), with a lack of a change in non-PDE4 cAMP hydrolyzing activity (t-test$_{(17)}$ = -0.77 P = 0.453; Fig. 1J). Increased hippocampal PDE4 activity caused reduced cAMP levels in the hippocampus (eGFP, n = 5; PDE4A5, n = 5; t-test$_{(7)}$ = 3.94, P = 0.0056; Fig. 2A), but not in either the prefrontal cortex (PFC) or cerebellum (CB) (t-test$_{(7)}$ = 0.35, P = 0.732, and t-test$_{(8)}$ = -1.16, P = 0.279; Fig. 2A). A reduction in cAMP-immunoreactivity was evident in all three major hippocampal regions (eGFP, n = 8; PDE4A5, n = 8; CA1, test$_{(14)}$ = 2.10, P = 0.029; CA3, t-test$_{(14)}$ = 2.50, P = 0.026; DG, t-test$_{(14)}$ = 2.51, P = 0.025; Fig. 2B,C), but not in the amygdala (AMY) (t-test$_{(14)}$ = -0.33, P = 0.372; Fig. 2B,C). Together, these data indicate that overexpression of PDE4A5 in hippocampal excitatory neurons increases PDE4 activity and reduces cAMP levels in the hippocampus without affecting cAMP content in other brain regions.

Elevated hippocampal PDE4A5 levels attenuate cAMP-dependent synaptic plasticity

Initial electrophysiological characterization of synaptic transmission in the Schaffer collateral-CA1 pathway involved measuring basal synaptic transmission and paired-pulse facilitation (PPF), an index of short-term plasticity. Increased PDE4A5 protein did not alter basal synaptic

transmission (eGFP, n = 8; PDE4A5, n = 8; t-test$_{(14)}$ = 1.37, P = 0.192; Fig. 3A) or PPF (eGFP, n = 9; PDE4A5, n = 9; ANOVA, effect of virus $F_{(1, 16)}$ = 0.024, P = 0.879; Fig. 3B). Maximum fEPSP slopes were not significantly different between groups (eGFP, n = 9, -10.56 ± 1.12 mV/ms; PDE4A5, n = 9, -10.45 ± 1.55 mV/ms; t-test$_{(12)}$ = 0.06, P = 0.949). Bath application of forskolin activates adenylyl cyclases leading to increased intracellular cAMP levels and synaptic potentiation (Vecsey et al., 2009; Havekes et al., 2012b; Park et al., 2014). PDE4A5 overexpression reduces the synaptic potentiation following application of forskolin (50 µM) with a significant reduction in the mean fEPSP slope over the last 20 minutes of the recording (eGFP, 175.7 ± 17.7%; PDE4A5, 109.4 ± 21.0%, t-test$_{(11)}$ = 2.42, P = 0.035; Fig. 3C). This finding suggests that cAMP-dependent synaptic plasticity in the hippocampus is impaired by elevating PDE4A5. In future studies, it will be interesting to determine whether PDE4A5 overexpression also reproduces deficits in other forms long-lasting LTP known to be attenuated by sleep deprivation such as LTP induced by spaced four-train or theta burst stimulation (Vecsey et al., 2009; Prince et al., 2014).

Increasing PDE4A5 in the hippocampus attenuates the formation of long-term context-shock associations

We first assessed whether selective overexpression of PDE4A5 in hippocampal neurons attenuates long-term memory formation of context-shock associations. Increased PDE4A5 protein in the hippocampus did not affect freezing levels during training (eGFP, n = 14; PDE4A5, n = 14; pre-shock, t-test$_{(26)}$ = 0.83, P > 0.40; post-shock, t-test$_{(26)}$ = -0.20, P > 0.84; Fig. 4A), but resulted in reduced freezing levels during re-exposure to the training context 24

hours after conditioning (eGFP, 41.9 ± 2.7%; PDE4A5, 29.3 ± 3.0%; t-test$_{(26)}$ = 3.06, P = 0.005; Fig. 4A). Because short-term contextual fear memories do not rely on cAMP signaling (Bourtchouladze et al., 1998), we hypothesized that PDE4A5 overexpression would not affect short-term memory formation for context-shock associations. Consistent with this, increasing PDE4A5 protein did not alter freezing levels during training (eGFP, n = 9; PDE4A5, n = 9; pre-shock, t-test$_{(16)}$ = -0.18, P = 0.85 post-shock, t-test$_{(16)}$ = 0.87, P = 0.39; Fig. 4B), or the retention test 1 hour after training (t-test$_{(16)}$ = 0.48, P > 0.85, P = 0.64; Fig. 4B). Thus, increasing PDE4A5 protein specifically affects the consolidation of long-term contextual fear memories.

Tone-cued fear conditioning is a task in which rodents associate a tone (conditioned stimulus, CS) with a mild electrical shock (US) a process that requires the amygdala rather than the hippocampus (LeDoux, 2000). Mice (eGFP, n = 12; PDE4A5, n = 13) overexpressing PDE4A5 expressed similar freezing levels during the conditioning (pre-shock, t-test$_{(23)}$ = 0.80, P = 0.43; CS; t-test$_{(23)}$ = 1.06, P = 0.30; post-shock, test$_{(23)}$ = 0.54, P = 0.60; Fig. 4C). Twenty-four hours after training, mice were exposed to a novel context and re-exposed to the CS. Both groups showed similar freezing levels during exposure to the novel context (t-test$_{(23)}$ = -0.66, P = 0.51; Fig. 4C), and exposure to the CS (t-test$_{(23)}$ = 0.26, P = 0.80; Figure 4C). Thus tone-cued fear conditioning is not affected by overexpression of PDE4A5 in hippocampal neurons.

PDE4A5 differs from other isoforms by virtue of a 102 amino acid unique N-terminal targeting domain (Beard et al., 2002; Bolger et al., 2003). To determine whether PDE4A5 requires this domain to target signaling complexes critical for the formation of context-shock associations, we engineered a truncated version of PDE4A5$^{\Delta 4}$ that lacks the first 303 base pairs encoding the isoform unique N-terminal domain (Bolger et al., 2003) (referred to as PDE4A5$^{\Delta 4}$) and virally expressed this N-terminally truncated species in hippocampal neurons. In contrast to

full-length PDE4A5, this truncated version failed to impair long-term memory for context-shock associations (t-test$_{(15)}$ = -1.10, P > 0.28; Fig. 4D). To further assess specificity of the PDE4A5-mediated long-term memory deficit, we replicated the experiment, but now increasing levels of the PDE4A1 isoform. This short PDE4A isoform is exclusively membrane associated, with a major fraction of PDE4A1 found compartmentalized to the Golgi (Shakur et al., 1995; Pooley et al., 1997). In contrast to increased expression of PDE4A5, elevated protein levels of PDE4A1 in hippocampal neurons did not affect long-term memory formations (t-test$_{(13)}$ = -1.25, P > 0.23; Fig. 4D). Together, these findings indicate that the unique N-terminal region of PDE4A5 confers the functional targeting of a core catalytic PDE4A module to a critical signaling complex in hippocampal excitatory neurons involved in regulating the formation of long-term contextual fear memories where it determines spatially localized cAMP degradation.

Overexpression of PDE4A5 in hippocampal excitatory neurons attenuates the consolidation of long-term object-place memories

To examine the impact of PDE4A5 overexpression on a non-aversive hippocampus-dependent task, we turned to the object-location memory paradigm in which mice have to learn and remember the location of individual objects (Oliveira et al., 2010; Havekes et al., 2014). Mice expressing eGFP (n = 14) or PDE4A5 (n = 15) gradually reduced object exploration levels across the training sessions indicating that animals acquired the locations of the individual objects (eGFP: session 1, 18.9 ± 1.3 s; session 2, 13.0 ± 1.2 s; session 3, 13.2 ± 2.0 s; PDE4A5: session 1, 24.1 ± 2.2 s; session 2, 18.4 ± 1.8 s; session 3, 13.7 ±1.7 s; ANOVA effect of session $F_{2,54}$ = 34.9, P = 0.0001). PDE4A5 mice showed overall slightly elevated exploration levels (ANOVA, effect of virus, $F_{1,27}$ = 4.756, P = 0.038). During the test session 24 hours after training, eGFP mice preferentially explored the displaced object indicating that they successfully remembered

the previous location of the individual objects. In contrast, mice overexpressing PDE4A5 explored all objects to a similar extent, indicative of a poor memory for the original object locations (eGFP, 49.8 ± 5.2%; PDE4A5, 31.3 ± 3.1%; t-test$_{(27)}$ = 3.11, P = 0.004; Fig. 4E).

The novel object recognition task (NOR) is a task that is based on the natural tendency of rodents to explore novel objects (Ennaceur and Delacour, 1988; Oliveira et al., 2010). Because the training conditions we use do not require an intact hippocampus (Oliveira et al., 2010), we anticipated that object-identity memories should not be affected by overexpression of PDE4A5 in hippocampal neurons. During training, eGFP (n = 8) and PDE4A5 (n = 10) mice showed no preference for either object (Fig. 4F) although the total object exploration levels were elevated in the mice overexpressing PDE4A5 (eGFP, 20.9 ± 1.1%; PDE4A5, 31.3 ± 3.1%; t-test$_{(16)}$ = -3.923, P <0.005). During the test session 24 hours after training, both groups preferentially explored the novel object indicating they successfully discriminated the novel from the familiar object (ANOVA, effect of object preference, $F_{1,16}$ = 18.623, P = 0.001; ANOVA, effect of virus, $F_{1,16}$ = 0.274, P = 0.608; interaction effect, $F_{1,16}$ = 0.013, P = 0.912; Fig. 4F).

To determine whether N-terminal-mediated targeting of PDE4A5 was essential for the ability of PDE4A5 to regulate the formation of object-place memories, we again expressed the N-terminal truncated form of PDE4A5, referred to as PDE4A5$^{\Delta 4}$, in hippocampal neurons and trained mice in the object-location memory task. During the retention test, then some 24 hours after training both groups preferentially explored the displaced object (eGFP, n = 8; PDE4A5$^{\Delta 4}$, n = 9; ANOVA, effect of object, $F_{1,15}$ = 25.217, P = 0.0001; ANOVA, effect of virus, $F_{1,15}$ = 0.36, P = 0.852; Fig. 4G). Next we assessed whether elevated expression of the short PDE4A isoform PDE4A1, that targets the Golgi (Shakur et al., 1995; Pooley et al., 1997), would similarly attenuate the formation of object-location memories. In line with our fear conditioning

results, increased expression of PDE4A1 did not affect the formation of object-location memories (eGFP, n = 8; PDE4A1, n = 7; ANOVA, effect of object, $F_{1,13} = 63.271$, $P = 0.0001$; ANOVA, effect of virus, $F_{1,13} = 0.277$, $P = 0.608$; Fig. 4G). The latter finding suggests that PDE4A1 does not target protein complexes critical for the formation of object-location memories. These data imply that PDE4A5 negatively impacts the formation of object-location memories by involving protein-protein interactions that target its unique N-terminal domain.

In a final set of behavioral studies, we determined whether the memory deficits associated with PDE4A5 expression could be a result of behavioral abnormalities unrelated to memory formation. Hippocampal overexpression of PDE4A5 does not affect explorative behavior or anxiety levels in respectively an open field (eGFP, n = 6; PDE4A5, n = 8; t-test$_{(12)} = -1.44$, $P = 0.175$; Fig. 4H) and zero maze (eGFP, n = 9; PDE4A5, n = 10; t-test$_{(17)} = 0.653$, $P = 0.523$; Fig. 4I). Thus, our behavioral studies indicate that the N-terminal targeting domain of PDE4A5 plays an essential role in the targeting of cAMP signaling critical for long-term memory formation.

Full length PDE4A5 but not truncated PDE4A5 attenuates forskolin-mediated FRET cAMP responses

Because our behavioral studies suggested that the N-terminal domain plays an essential role in the compartmentalization of PDE4A5, we measured cAMP responses using the ICUE3 biosensor in hippocampal neurons expressing either a control vector, full-length PDE4A5, or the N-terminal lacking PDE4A5$^{\Delta 4}$. Baseline FRET responses were not affected by overexpression of either construct (ANOVA, effect of construct, $F_{2,44} = 1.984$, $P = 0.198$; Figure 5A, B).

Expression of PDE4A5 but not PDE4A5$^{\Delta 4}$ attenuated the forskolin-mediated FRET response (ANOVA, effect of construct, $F_{2,44} = 11.991$, $P < 0.0001$. PDE4A5 vs control Tukey test $P < 0.001$, PDE4A5 vs PDE4A5$^{\Delta 4}$ Tukey test $P = 0.001$; Figure 5A, B). Consecutive bath application with the PDE inhibitor IBMX normalized the FRET responses (ANOVA, effect of construct, $F_{2,44} = 0.295$, $P = 0.746$; Figure 5A, B), indicating that the decrease in FRET response due to overexpression of PDE4A5 was not due to non-specific alterations in PDE/cAMP signaling. Together these data indicate that the N-terminal of PDE4A5 plays a critical role in the localization of PDE4A5 to specific intracellular domains where it hampers local cAMP-dependent processes critical for synaptic plasticity and memory.

The N-terminal domain of PDE4A5 plays an essential role in the compartmentalization of PDE isoforms

Our behavioral data suggest that the N-terminal domain of PDE4A5 plays an essential role in the compartmentalization of the PDE4A5 isoform to cAMP-containing complexes critical for learning and memory. Therefore, we virally expressed full-length and truncated N-terminal-lacking PDE4A5 in cultured hippocampal neurons to compare the intracellular distribution of these isoforms. We found that the focus of full-length PDE4A5 immunofluorescence in the cultured neurons is at both a discrete perinuclear area and in dendritic compartments (Left panels, Figure 6). In contrast, truncated PDE4A5 lacking the N-terminal domain unique for this isoform was located predominantly in the perinuclear region (Middle panels, Figure 6). These findings confirm previous work highlighting the importance of isoform unique N-terminal domains in determining the intracellular distribution of PDE4A enzymes (Houslay et al., 1998; Huston et al., 2000).

Discussion

Phosphodiesterases play an essential role in orchestrating the compartmentalized degradation of cAMP leading to local changes in cAMP signaling in specific subcellular domains in the cell (Houslay, 2010). This is in part achieved by isoform-specific N-terminal domains that bind to specific localized protein complexes, which enables individual PDE isoforms to target local cAMP signaling and thereby regulate a unique set of molecular processes (Houslay, 2010). The PDE4A5 isoform has a unique 102 amino acid N-terminal region involved in its intracellular targeting (Beard et al., 2002; Bolger et al., 2003). Using a viral approach, we show that increased expression of PDE4A5 selectively in hippocampal excitatory neurons attenuates a cAMP-dependent form of synaptic plasticity in hippocampal area CA1, reduces forskolin-mediated increases in cAMP content in cultured hippocampal neurons, and impairs long-term memory formation specifically in learning tasks that require the hippocampus. Furthermore, to assess the role of the N-terminal domain of PDE4A5 in compartmentalizing and targeting cAMP-dependent signaling complexes critical for memory storage, we replicated the biochemical and behavioral studies using an N-terminal lacking truncated version of the same protein. In contrast to the observations with full-length PDE4A5, expression of the N-terminal truncated PDE4A5 construct does not attenuate forskolin-mediated alterations in cAMP responses in cultured neurons, does not affect hippocampus-dependent forms of memory but does alter the intracellular distribution of this isoform.

Our overexpression studies with full-length PDE4A5 and PDE4A1 as well as truncated PDE4A5 emphasize that increases in protein levels of specific PDE4 isoforms initiates the decrease in cAMP levels in specific neuronal compartments rather than causing a global non-specific decrease in cAMP throughout the cell. Our overexpression studies suggest that the

common domains shared by the different PDE4A isoforms, including the catalytic domain and the conserved regulatory regions 1 and 2 (UCR1 and UCR2), do not likely contribute to the hippocampus-dependent cognitive deficits associated with PDE4A5 overexpression. The lack of an impact of the N-terminal lacking PDE4A5 isoform at the behavioral level is not likely to be explained by a reduction in the efficacy of the truncated isoform to degrade cAMP as we previously showed that the loss of the isoform-specific N-terminal domain leads to an increase rather than decrease in catalytic activity (Beard et al., 2002).

Our findings also reveal the importance of the isoform-specific N-terminal domain for targeting individual PDE4 isoforms to cAMP signaling critical for cognitive processes and confirms and expands on previous *in vitro* work showing that truncation of the N-terminal region of PDE4A5 alters its membrane association, and localization at the cell margin (Huston et al., 2000; Beard et al., 2002; Bolger et al., 2003; Houslay, 2010). At the membrane, PDE4 isoforms can interact with molecular elements critical for learning and memory such as beta arrestins (Li et al., 2009). The unique N-terminal domain of PDE4A5 also allows for association with certain SH3 domain containing proteins such as certain members of the src tyrosyl kinase family; including Src, Fyn, and Lyn (Beard et al., 1999; Beard et al., 2002), and recent work indicates that Fyn inhibition reverses memory deficits in mouse models for Alzheimer's disease (Kaufman et al., 2015). PDE4A5 also interacts with Disrupted-in-Schizophrenia 1 (DISC1) (Murdoch et al., 2007), a major risk factor for schizophrenia and other psychiatric diseases such as bipolar disorder and depression (Porteous and Millar, 2006). Therefore misregulation of DISC1-related signaling mechanisms may contribute to the endophenotypes associated with overexpression of PDE4A5. The N-terminal domain of PDE4A5 also binds the immunophilin XAP2 (also called AIP and ARA9) (Bolger et al., 2003), although to our knowledge no studies have examined the

role of XAP2 in learning and memory. Deletion of the unique part of the N-terminal domain also disrupts the focus of PDE4A5 localization within ruffles at the cell margin (Beard et al., 2002). As membrane ruffles play an essential role in cell motility and spine dynamics and through the remodeling of the actin cytoskeleton (Chhabra and Higgs, 2007; Honkura et al., 2008), one additional mechanism through which PDE4A5 could affect memory formation is by negatively impacting cAMP-dependent signaling mechanisms that modulate actin dynamics. Future studies including co-immunoprecipitation and co-localization experiments with the N-terminal domain binding proteins will have to define whether these candidate mechanisms indeed contribute to the plasticity deficits and memory impairments associated with elevated expression of this isoform.

Sleep deprivation negatively impacts cognitive processes, particularly those that require the hippocampus (Havekes et al., 2012a; Abel et al., 2013; Kreutzmann et al., 2015). We previously found that a single brief period of sleep deprivation elevated PDE4 activity and reduced cAMP levels in the hippocampus. These changes were accompanied by a specific increase in PDE4A5 protein expression without affecting the protein levels of other PDE4 isoforms such as PDE4B, PDE4D3, and PDE4D5 (Vecsey et al., 2009). The memory deficits associated with a single 5-hour period of sleep deprivation could be prevented by pharmacological inhibition of the PDE4 family (Vecsey et al., 2009), and transiently increasing cAMP levels selectively in hippocampal neurons (Havekes et al., 2014). Despite these observations, it remains to be defined whether the increase in PDE4A5 function in the hippocampus was causally related to the memory and plasticity impairments associated with sleep loss. In our current study, we observed that increasing hippocampal PDE4A5 levels impairs long-term memory formation in contextual fear conditioning and object-place

recognition tasks (Fig. 4). In both learning paradigms, the formation of long-term memories critically depends on proper hippocampal function (LeDoux, 2000; Oliveira et al., 2010), and the observed impairments in both tasks mimic those observed with sleep deprivation (Graves et al., 2003; Vecsey et al., 2009; Hagewoud et al., 2010; Florian et al., 2011; Havekes et al., 2012a; Abel et al., 2013; Havekes et al., 2014). Using electrophysiological recordings, we show that increasing PDE4A5 protein levels impairs forskolin-induced synaptic plasticity (Fig. 3), a deficit that also occurs after 5 hours of sleep deprivation (Vecsey et al., 2009). In summary, this work reveals that elevated expression of the PDE4A5 isoform in hippocampal neurons is sufficient to mimic electrophysiological, and cognitive phenotypes associated with sleep loss.

To our knowledge, this work is the first to define the function of the PDE4A5 isoform and protein domains in hippocampal function. From a broader perspective, our work will help define the contribution of individual PDE isoforms to the endophenotypes of neurocognitive and neuropsychiatric disorders that are accompanied by altered PDE signaling and facilitate the development of novel therapeutic strategies based on the targeting of specific domains of individual PDE isoforms. Related to this notion, there is now a wealth of evidence consistent with the idea that the evolutionary conserved diversity of N-terminal regions that define specific PDE4 isoforms is related to their recruitment to specific signaling complexes (Houslay, 2010). We, and others, have demonstrated that such interactions can be disrupted using cell permeable peptides and also that such species can phenocopy dominant negative approaches (*i.e. (Smith et al., 2007; Serrels et al., 2010; Martin et al., 2014)*. Indeed, an engineered peptide disrupting PDE4D-HSP20 interactions shows in vivo efficacy in a mouse model of pressure overload mediated hypertrophy (Martin et al., 2014). The ability to refine such disruptor peptides for in vivo use could be facilitated through use of NMR to uncover their structure (Smith et al., 2007)

together with the incorporation of non-native amino acids and the addition of lipid species to facilitate cell entry together with truncation and modification to generate peptidomimetic species. Identification of the functional anchor species for PDE4A5 in this scenario would then take developments to a level where high-throughput screening for small molecule, drug-like disruptor species of PDE4A5 and itAs functional anchor becomes a realistic approach. Additionally, high content screens for compounds that dislocate PDE4A5 from its relevant compartment in cells would provide another means of such compounds. The aim would be to uncover small peptides and peptidomimetics of small molecules that enter the brain and dislocate PDE4A5 from the functionally relevant anchor protein / signaling complex in order to recapitulate the phenotype we describe in these studies.

Figure Legends

Figure 1. Overexpression of PDE4A5 in hippocampal excitatory neurons increases PDE4A5 protein levels and PDE4 activity. ***A***, Mice were bilaterally injected with AAV_9-CaMKII-eGFP or AAV_9-CaMKII-PDE4A5-VSV into the hippocampus. ***B***, Viral PDE4A5 protein expression was restricted to the hippocampus. ***C-E***, Viral expression of PDE4A5 was observed in all three major hippocampal subregions. ***F-H***, Transgene expression was not observed in hippocampal astrocytes (PDE4A5-VSV red, GFAP green; lower panels). ***I***, A representative immunoblot for PDE4A5 protein levels. Viral overexpression of PDE4A5 leads to significantly higher PDE4A5 protein levels in hippocampal lysates ($n = 4$; $t\text{-test}_{(7)} = -6.84$, $P = 0.001$). ***J***, Overexpression of PDE4A5 significantly increases PDE4 activity ($n = 9\text{-}10$, $t\text{-test}_{(17)} = 13.67$, $P = 0.0001$) without affecting non-PDE4 activity ($t\text{-test}_{(17)} = -0.77$ $P = 0.453$). All error bars denote s.e.m. *** indicates $P = 0.001$, **** indicates $P = 0.0001$.

Figure 2. Overexpression of PDE4A5 in hippocampal excitatory neurons reduces cAMP levels in the hippocampus. ***A***, Viral expression of PDE4A5 reduces cAMP levels in the hippocampus (eGFP, $n = 5$; PDE4A5, $n = 5$; $t\text{-test}_{(7)} = 3.94$, $P = 0.0056$), but not in the prefrontal cortex ($t\text{-test}_{(7)} = 0.35$, $P = 0.732$), or cerebellum ($t\text{-test}_{(8)} = -1.16$, $P = 0.279$). ***B***, ***C*** Representative cAMP immunoreactivity images in mice expressing eGFP or overexpressing PDE4A5. Note the reduction in cAMP immunoreactivity in the major hippocampal subregions (eGFP, $n = 8$; PDE4A5, $n = 8$; CA1, $t\text{-test}_{(14)} = 2.10$, $P = 0.0291$; CA3, $test_{(14)} = 2.50$, $P = 0.026$; DG, $test_{(14)} = 2.51$, $P = 0.025$), but not in the amygdala ($n = 8$, $t\text{-test}_{(14)} = -0.33$, $P = 0.372$). All error bars denote s.e.m. * indicates $P < 0.05$, ** indicates $P = 0.01$.

Figure 3. Overexpression of PDE4A5 in hippocampal excitatory neurons reduces forskolin-induced potentiation. ***A***, Input-output curves relating the amplitude of the presynaptic fiber volley to the initial slope of the corresponding fEPSP at various stimulus intensities was not altered due to viral overexpression of PDE4A5 in hippocampal neurons (n = 8, $t\text{-test}_{(14)} = 1.37$, $P = 0.192$). ***B***, PDE4A5 overexpression does not change paired-pulse facilitation, a short-term form of synaptic plasticity, in hippocampal slices (n = 9, two-way repeated measures ANOVA, effect of virus $F_{(1, 16)} = 0.024$, $P = 0.879$). ***C***, Elevated expression of PDE4A5 in hippocampal neurons attenuates LTP induced by bath application with the adenylate cyclase activator forskolin. The mean fEPSP slope over the last 20 minutes of the recording was significantly reduced in PDE4A5 mice (eGFP, n = 5, 175.7 ± 17.7%; PDE4A5, n = 8, 109.4 ± 21.0%, $t\text{-test}_{(11)} = 2.42$, $P = 0.0253$). In all sample sweeps, black traces indicate baseline, and red traces were acquired at 1 hr after tetanus.

Figure 4: Localization of PDE4A5 by the unique N-terminal plays a central role in the memory deficits associated with overexpression of PDE4A5. ***A***, Overexpression of PDE4A5 in hippocampal neurons impairs the formation of long-term memories for context fear associations (n = 14 for both groups, $t\text{-test}_{(26)} = 3.06$, $P = 0.005$) without affecting freezing levels during training (pre-shock, $t\text{-test}_{(26)} = 0.83$, $P = 0.40$; post-shock, $t\text{-test}_{(26)} = -0.20$, $P = 0.84$). ***B***, In contrast, elevated PDE4A5 protein levels in hippocampal neurons do not change the formation of short-term contextual fear memories (n = 9, t-test, pre-shock, $t\text{-test}_{(16)} = -0.18$, $P = 0.85$ post-shock, $t\text{-test}_{(16)} = 0.87$, $P = 0.39$). ***C***, PDE4A5 overexpression in hippocampal neurons does not alter the consolidation of amygdala-dependent tone-cued fear memories (n = 12-13, Pre-CS, $t\text{-test}_{(23)} = -0.66$, $P = 0.51$; CS, $t\text{-test}_{(23)} = 0.26$, $P = 0.80$) and did also not affect freezing level during training in either fear conditioning paradigm ($P > 0.3$ for all comparisons). ***D***,

Overexpression of a truncated form of PDE4A5, PDE4A5$^{\Delta4}$, that lacks the isoform unique N-terminal domain, or the short isoform PDE4A1 in hippocampal neurons does not alter the formation of long-term contextual fear memories (eGFP vs PDE4A5 $^{\Delta4}$, n=7-9, t-test$_{(15)}$ = -1.10, P = 0.28; eGFP vs PDE4A1, n = 7-9, t-test$_{(13)}$ = -1.25, P > 0.23; Fig. 4D). ***E***, Increasing expression of PDE4A5 in hippocampal excitatory neurons impairs memory consolidation for object-location (n = 14-15, t-test$_{(27)}$ = 3.11, P = 0.004). ***F***, In contrast, increasing PDE4A5 protein levels in the hippocampus leaves the consolidation of long-term memories for object identity undisturbed (n = 8-10, ANOVA, effect of object preference, $F_{1,16}$ = 18.623, P = 0.001; two-way ANOVA, effect of virus, $F_{1,16}$ = 0.274, P = 0.608; interaction effect, $F_{1,16}$ = 0.013, P = 0.912). ***G***, Overexpression of the N-terminal lacking PDE4A5$^{\Delta4}$ or the short isoform PDE4A1 in hippocampal neurons does not modulate memory consolidation for object location (n = 7-9, eGFP vs PDE4A5 $^{\Delta4}$, eGFP vs PDE4A1, t-test, two-way ANOVA P > 0.42 for both comparisons). ***H***, ***I***, Elevated expression of PDE4A5 in hippocampal excitatory neurons does not alter exploratory behavior in an open field (n = 6-8, t-test$_{(12)}$ = -1.44, P = 0.175) or anxiety levels in the zero maze (n = 9-10, t-test$_{(17)}$ = 0.653, P = 0.523. The dotted line indicates no preference. All error bars denote s.e.m. ** indicates P = 0.01 *** indicates P = 0.005.

Figure 5. Full-length but not truncated PDE4A5 attenuates forskolin-mediated cAMP responses assessed by a FRET indicator. ***A***, FRET cAMP responses were measured in cultured hippocampal neurons expressing a control construct, PDE4A5, or truncated N-terminal lacking PDE4A5$^{\Delta4}$ after consecutive bath application with the adenylate cyclase activator forskolin and phosphodiesterase inhibitor IBMX. Forskolin treatment significantly elevated cAMP levels in control neurons and neurons expressing PDE4A5$^{\Delta4}$ whereas the cAMP response was attenuated in neurons overexpressing full-length PDE4A5. ***B***, Average FRET sensor

responses for the different treatment conditions. Baseline FRET responses were not affected by overexpression of either construct (n = 11-20, ANOVA, effect of construct, $F_{2,44} = 1.984$, $P = 0.198$). Expression of PDE4A5 but not PDE4A5$^{\Delta 4}$ attenuated the forskolin-mediated FRET response (n = 11-20, ANOVA, effect of construct, $F_{2,44} = 11.991$, $P < 0.0001$. PDE4A5 vs control Tukey test $P < 0.001$, PDE4A5 vs PDE4A5$^{\Delta 4}$ Tukey test $P = 0.001$). Consecutive bath application with the PDE inhibitor IBMX normalized the FRET responses (ANOVA, effect of construct, $F_{2,44} = 0.295$, $P = 0.746$). CT =20 cells from 3 preparations; PDE4A5 = 11 cells from 3 preparations; PDE4A5$^{\Delta 4}$ = 14 cells from 3 preparations. All error bars denote s.e.m. *** indicates $P \leq 0.001$.

Figure 6. Loss of the isoform-unique N-terminal domain alters the compartmentalization of PDE4A5 in hippocampal neurons. Full-length PDE4A5 is compartmentalized to both a discrete perinuclear area and dendritic compartments (left panels). In contrast, truncated PDE4A5 lacking the N-terminal domain unique for the isoform was located predominantly in the perinuclear region (middle panels). PDE4A5 expression is absent from the nucleus (right panels).

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Figure 1, Havekes et al

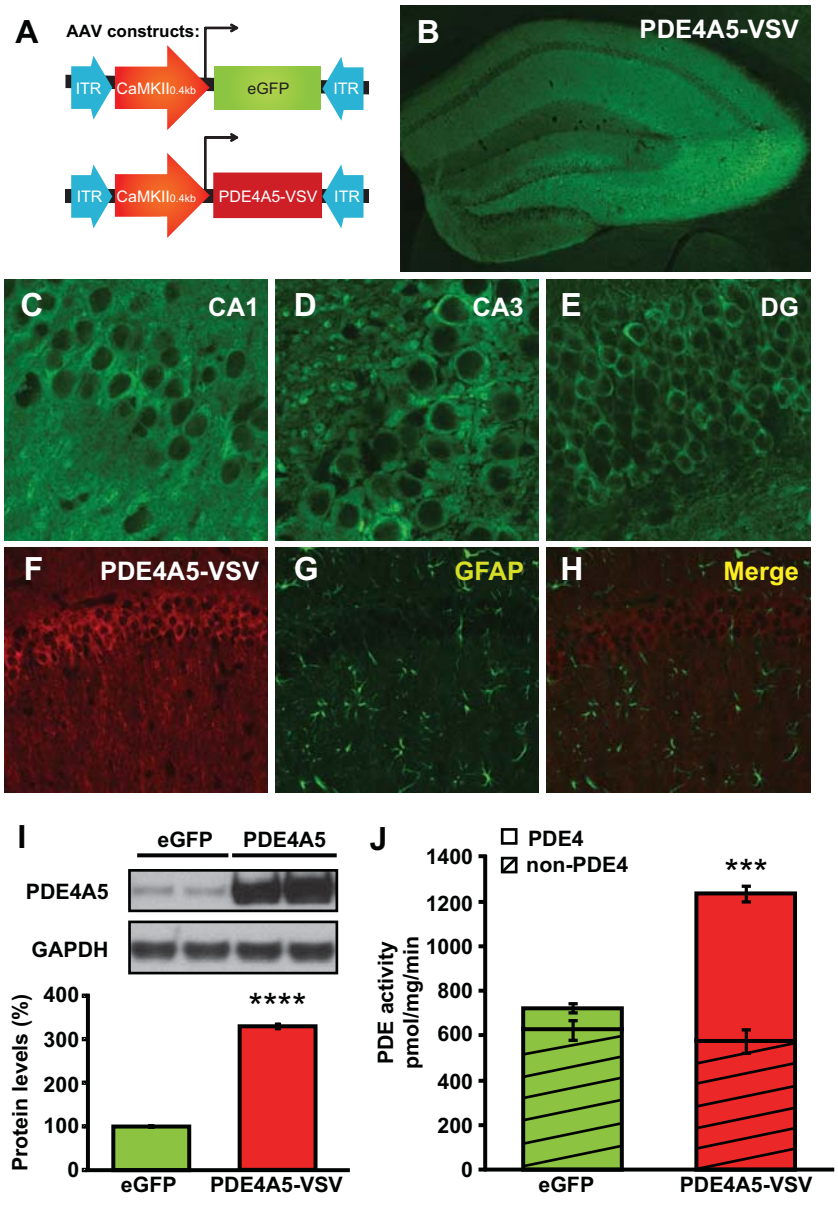
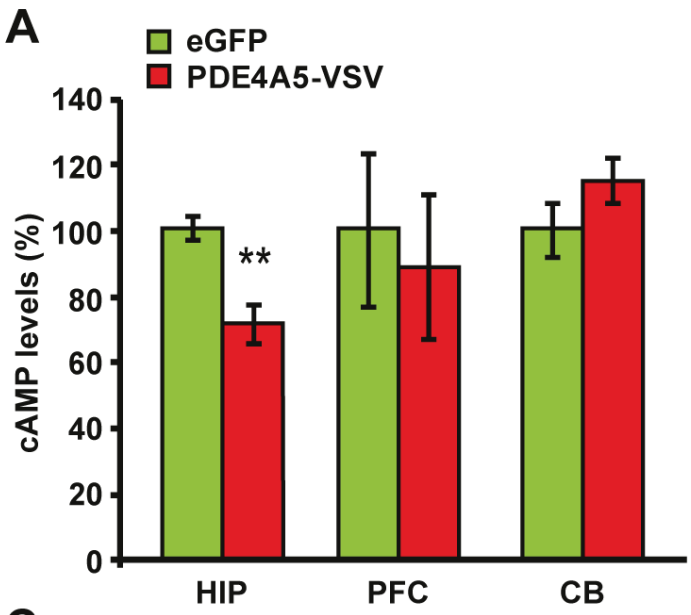
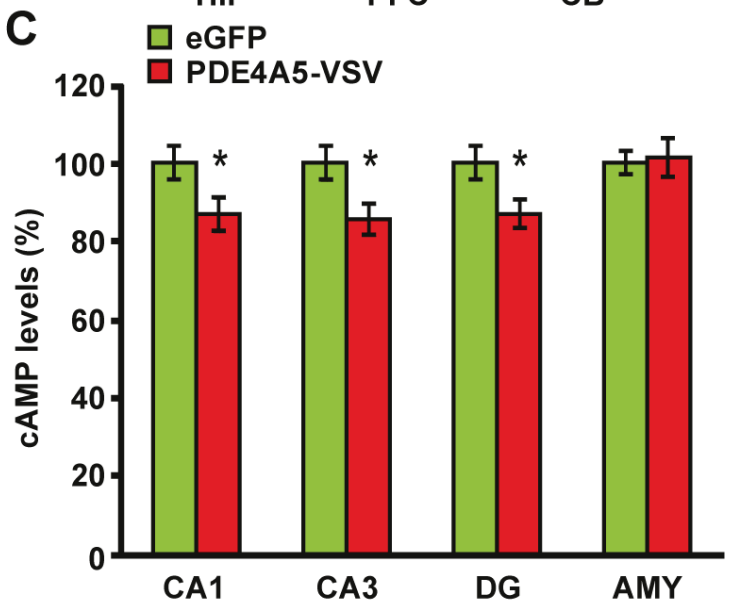


Figure 2, Havekes et al





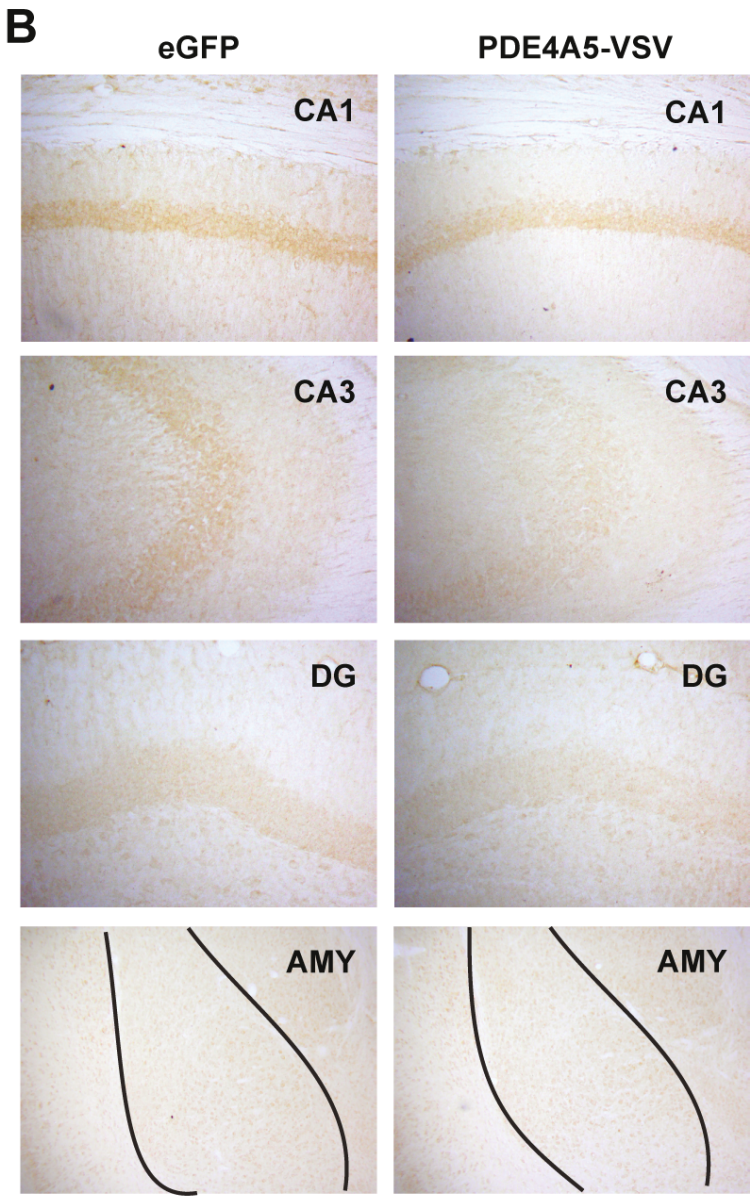
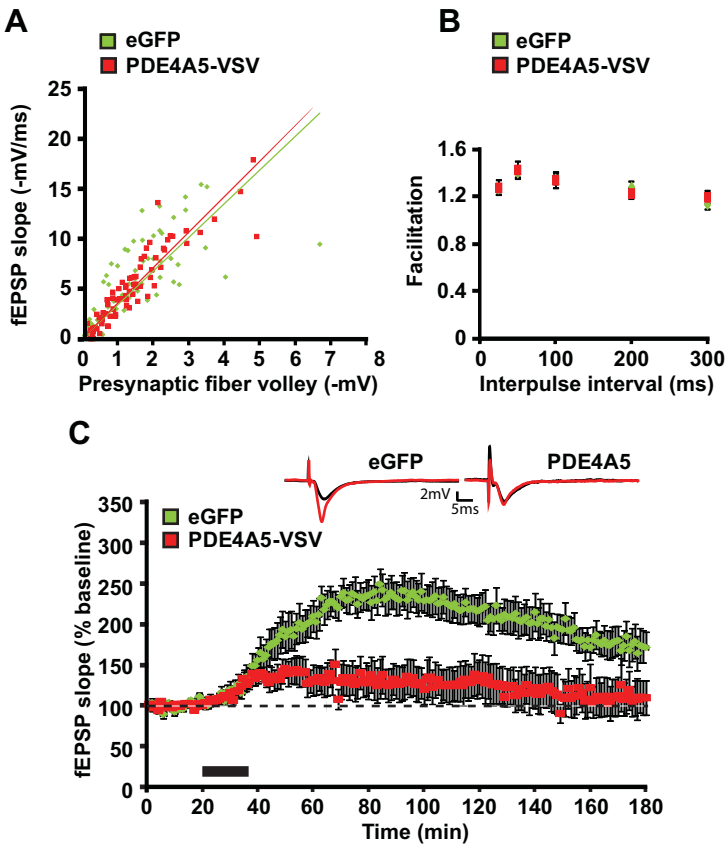


Figure 3, Havekes et al



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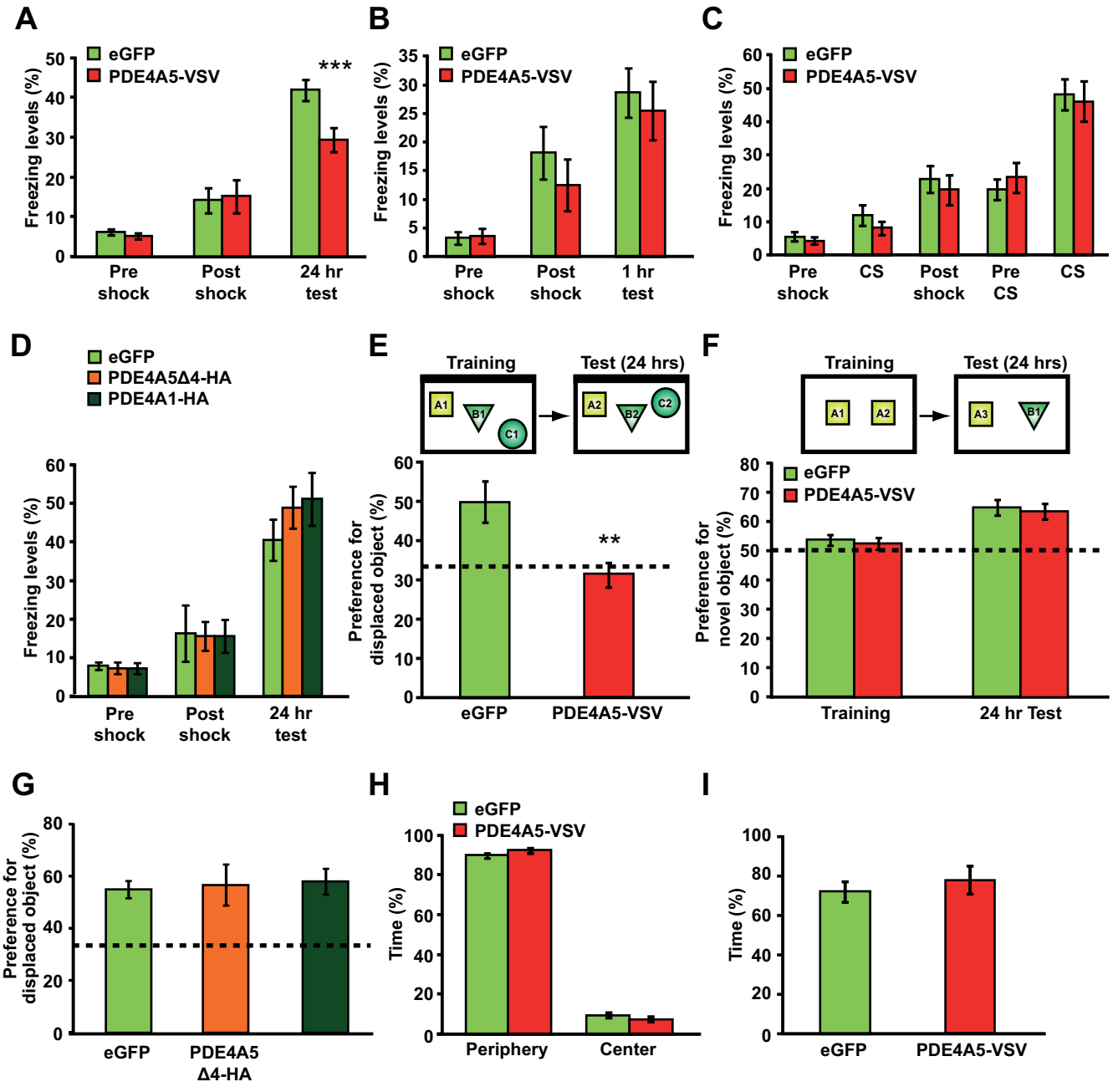
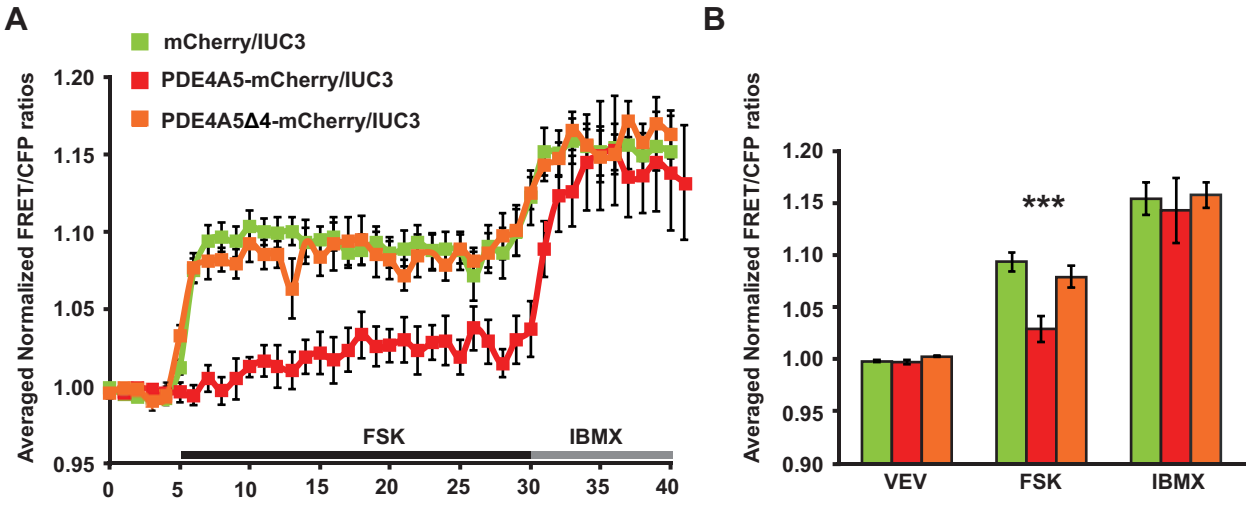


Figure 5, Havekes et al



Havekes et al Figure 6